

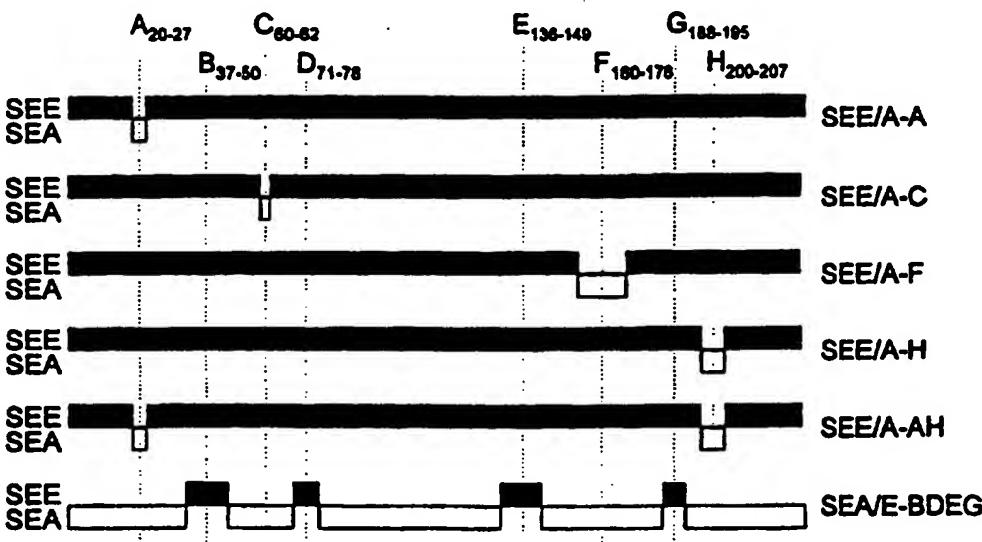


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(54) Title: MODIFIED/CHIMERIC SUPERANTIGENS AND THEIR USE



(57) Abstract

A conjugate between a target-seeking moiety and a modified superantigen, characterized in that the superantigen is a wild-type superantigen (SA I) in which an amino acid residue in a superantigen region (region I) determining binding to TCR, preferably TCRV β , and T cell activation have been replaced by another amino acid residue while retaining the ability to activate a subset of T cells. In a preferred embodiment the modified superantigen is a chimera between at least two wild-type superantigens (SA I, SA II etc.) characterized in that one or more amino acid residues in a region determining binding to TCR and T cell activation have been interchanged between various wild-type superantigens. A therapeutic method making use of modified/chimeric superantigens as defined in the preceding paragraphs. An antibody preparation in which the cysteine residues that provide for interchain disulfide bonds have been mutated so as to forbid interchain disulfide bridges, preferably to serine residues, for use as a pharmaceutical.

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MODIFIED/CHIMERIC SUPERANTIGENS AND THEIR USE

This application claims priority from Swedish Patent Application No 9601245-5, which was filed March 29, 1996, and 5 from US application 08/695,692, which was filed August 12, 1996, both of which are incorporated herein by reference.

Field of the invention

The present invention relates to functionally active modified 10 superantigens which are wild-type superantigens (SA I) in which one or more amino acid residues have been substituted while maintaining superantigen function. In case one or more of the 15 substituting residues (or a conserved amino acid residue thereof) occur in the corresponding positions in another wild-type superantigen (SA II), the modified superantigen is called a chimera (sometimes hybrid). Chimeric superantigens thus will contain part sequences/regions deriving from at least two different wild-type superantigens.

By the term "corresponding" is meant that residues, part 20 sequences and regions replacing each other have functionally the same position in superantigens I and II so that substitution will lead to a chimeric form that is able to function as a superantigen.

The terminology grafted/grafting/graft is used in connection 25 with parts of the full sequence of superantigen II that have replaced corresponding parts of superantigen I, even if only one single amino acid has been replaced.

Modified/chimeric superantigens also encompass functional 30 superantigens modified in other ways, for instance modified by amino acid replacements in regions other than those related to the invention, conjugated to a target-seeking moiety including also fused forms when the moiety is a polypeptide/protein. The order for carrying out the modifications may vary. See below.

SUPERANTIGENS

According to the very first definition (around 1988-1993), superantigens are bacterial or viral proteins capable of binding to MHC class II antigens without prior intracellular processing and activate T cells by binding to the β -chain variable region ($V\beta$) of the T cell receptor (TCR). The binding leads to a $V\beta$ family restricted activation of a relatively large proportion/subset of T cells and lysis of MHC Class II expressing cells (superantigen dependent cell-mediated cytolysis = SDCC).

Well known wild-type superantigens according to the definition above are the staphylococcal enterotoxins (SEA, SEB, SEC1, SEC2, SED, SEE and SEH). Further examples are Toxic Shock Syndrome Toxin 1 (TSST-1, also of staphylococcal origin), Exfoliating Toxins (EXft), Streptococcal Pyrogenic Exotoxin A, B and C (SPE A, B and C), Mouse Mammary Tumor Virus proteins (MMTV), Streptococcal M proteins, Clostridial Perfringens Enterotoxin (CPET), mycoplasma arthritis superantigens etc. For a review of superantigens and their properties see Kotzin et al 1993.

During the early nineties it was discovered that activation and subsequent cell lysis could occur in a MHC class II independent manner in case the superantigen was conjugated with a target-seeking moiety capable of binding to a cell surface structure (Dohlsten et al WO9201470 and Abrahmsén et al WO9601650). Upon incubation of target cells (carrying the target structure for the target-seeking moiety) and effector cells (T cells) with the conjugates, the target cells become lysed (superantigen antibody dependent cell-mediated cytolysis = SADCC) without any requirement for class II expression.

Accordingly the superantigen concept of today and used in the context of the present invention, if not otherwise specified, encompasses any compound (preferably of polypeptide structure) that is capable of binding to a cell surface structure (target structure) and to one or more polymorphic TCR chain, in particular the $V\beta$ chain, thereby activating a subset of T cells

expressing the specific TCR chain involved in the binding. The T cells then become cytotoxic for cells carrying the surface structure (target structure, target cells). Normally the activated subset of T cells constitutes about 1-20% of the 5 total amount of T cells of an individual.

BACKGROUND ART - STRUCTURAL AND FUNCTIONAL STUDIES UTILIZING MUTATED AND CHIMERIC SUPERANTIGENS.

Chimeric superantigens including point mutated forms have 10 previously been described (Kappler et al WO 9314364, Kappler et al 1992; Grossman et al 1991; Hufnagle et al 1991; Hartwig et al 1993; Fraser et al 1993; Mollick et al 1993; Irwin et al 1992; Hudson et al 1993; and Blanco et al 1990). Mollick et al and Hudson et al show from studies of chimeras that the V β 15 specificity of SEA and SEE resides in certain amino acid sequences present in the carboxy terminal region (i.e. amino acid residues 200, 206 and 207). In addition to the V β specificity, mainly depending on this region, Mollik et al also were able to show that for complete reconstitution of SEE like 20 activity of SEA containing SEE grafts towards V β 8, a fragment containing the N-terminal 70 amino acid residues from SEE was needed. This fragment contains parts of the SEE-like MHC class II α chain binding site and chimeric SEA/SEE molecules containing this part from SEE, inhibited binding of SEA to MHC 25 class II DR1 in a SEE-like manner.

Recently SEE-SEA chimeras involving an exchange of regions involved in binding to TCRV β have been described (Lamphaer et al., J. Immunol. 156 (March 15, 1996) 2178-2185). A SEE superantigen Fab antibody fusion protein in which the SEE 30 domains involved in the interaction with T cells have been replaced with the corresponding non-homologous SEA domains has been discussed at ABRF'96: Biomolecular Techniques, Holiday Inn Golden Gateway, San Francisco, California March 30 - April 2, 1996 (Björk et al., M45).

BACKGROUND ART - THERAPEUTIC USE OF SUPERANTIGENS

Non-conjugated superantigens have been suggested for therapy with curative effect presumably being accomplished through a general activation of the immune system (Kalland et al 5 WO9104053; Terman et al WO9110680 and WO9324136; Newall et al 1991).

It has also been suggested to use modified superantigens conjugated to target-seeking moieties (Dohlsten et al WO9201470; Abrahmsén et al WO9601650, both hereby being 10 incorporated by reference). This enabled a broader therapeutic use of T cell activation through $V\beta$. The conjugates studied so far have had a diminished class II affinity, which in turn has lead to a decrease of the severe systemic toxicity normally associated with the wild-type superantigens.

15 Terman et al (WO9110680; WO9324136) in side-sentences suggested cancer therapy with modified superantigens and superantigen fragments.

Kappler et al (WO9314634) have suggested to use non-conjugated superantigens (SEB) mutated to have lost their $V\beta$ -binding or MHC Class II binding ability (in the context of 20 vaccines and to neutralize toxic effects of superantigens). Abrahmsén et al (WO9601650) have suggested cancer therapy with 25 conjugated superantigens having a modified, preferably decreased, ability to bind to Class II antigens. The modifications encompassed single mutations as well as construction of chimeras between different superantigens.

THE PROBLEMS THAT HAVE BEEN THE OBJECTIVE TO SOLVE WITH THE PRESENT INVENTION.

The sera of human populations normally contain high titers of 30 antibodies against superantigens. For the staphylococcal superantigens, for instance, the relative titers are TSST-1 > SEB > SEC1 > SE3 > SEC2 > SEA > SED > SEE. These relative titers indicate immunogenicity problems and problems with 35 neutralizing antibodies in case SEs are administered parenterally. Based solely on these problems, SEE should be the preferred staphylococcal superantigen. In the context of work

with fusion proteins, however, we have found that the ability for T cell MHC class II independent cytotoxicity, superantigen-antibody dependent cell cytotoxicity (SADCC), of SEE conjugates is poor. The anti-SE titers also indicate that there might be 5 advantages in modifying a "high titer" superantigen to be more like a "low titer" superantigen.

THE OBJECTIVES OF THE PRESENT INVENTION.

A first objective is to improve the previously known 10 superantigens with respect to lowering their immunogenicity and reaction with neutralizing antibodies.

A second objective is to provide superantigens with less side effects when used as a drug.

A third objective is to provide improved superantigens that 15 can be used as the active principle in the treatment of mammals suffering from cancers, autoimmune diseases, parasitic infestations, viral infections or other diseases associated with cells that on their surface express MHC class II antigens and/or structures that are specific for respective disease and 20 bind to a target-seeking moiety incorporated into the superantigen.

THE DISCOVERY THAT HAS RESULTED IN THE INVENTION

A sequence homology analysis of SEA and SEE (Fig 2) reveals 25 that the non-identical amino acid residues are concentrated to eight distinct regions. Outside these eight regions, making up to 34% of the sequence, the identity of the two SEs is 97%, with conserved amino acid substitutions accounting for the remaining differences. Four of these regions are structurally 30 close to the two MHC class II binding sites (B: AA 37-50, D: 71-78, E: 136-149, and G 189-195), and are not likely to interact with the TCR. The additional four regions (A: AA 20-27, C: 60-62, F: 161-176 and H:200-207) are located on the edge of the molecule, in the vicinity of the putative TCR binding 35 site, postulated to reside in the groove between the two subdomains. By grafting the individual regions (replacement of

amino acid residues that differ), we have now found that the property of SEA-conjugates to induce a cytotoxic response as well as potentiating proliferative response in the absence of MHC class II, resides in one region in the TCR binding domain of SEA. This Region (A) is transferable to SEE and has a great impact on activity in the absence of Class II, although limited effects on the V β specificity of the superantigen (Fig 6, Tab.2). All of the regions (A, C, F and H) seem to participate, directly or indirectly, in the interaction with the TCR manifested by an altered stimulatory effect on murine T-cell hybridomas (Tab. 2)

Due to the analogous mode of action it is conceivable that a similar structural separation of these TCRV β binding properties is at hand also for superantigens analogous to SEA and SEE. The same may also apply within other types of superantigens, in which the binding structures are organised differently. Our discovery has enabled us to outline the construction of chimeric superantigens that potentially are of extremely great value as therapeutic agents.

20

THE INVENTION

The first aspect of the invention is a method for the treatment of a disease in a mammal by activation of its immune system through administration of a therapeutically effective (immune activating) amount of a modified, preferably chimeric, superantigen. The mammal is preferably a human. The diseases in question are mostly associated with cells expressing on their surface a target structure binding to the superantigen. The target structure is in most cases different from the TCR epitope normally binding to superantigens. Binding to the target structure permits also binding to TCR and T cell activation. Illustrative examples are MHC class II antigens and other cell surface structures that may be expressed on cells associated with the courses of diseases. Illustrative diseases are malignant tumors including any type of cancers (such as carcinoma, sarcoma, melanoma, lymphoma etc.), viral infections,

parasitic infestations and autoimmune diseases. The cancer to be treated may be located to the colon, breast, cervix, kidney, stomach, small intestines, duodenum, prostate, testis, skin, lung, liver, pancreas, skeleton etc including also metastasis at various locations. The inventive active agent is also applicable to so called multi-drug resistant forms of cancers. The cells expressing the target structure may also be cells that in some way control or regulate the development of the disease to be treated.

10 The characteristic feature of the method is that one employs a modified superantigen in which one or more amino acid residues in a region (region I) providing for binding to a subset of T cells via a polymorphic TCR chain, in particular TCRV β , in a wild-type superantigen (SA I) has been replaced 15 with a respective amino acid residue retaining superantigen activity to the so modified superantigen. The presently preferred embodiments refer to a chimeric superantigen in which one or more amino acid residues in a region (region I) of a first wild-type superantigen (SA I) have been replaced with the 20 corresponding one or more amino acid residues in a corresponding region (region II) of a second wild-type superantigen (SA II). The regions I and II differ with respect to amino acid sequences. The superantigens I and II have been selected so that the regions I and II can replace each other 25 without killing the superantigen function. In this context one has to account for the fact that a certain region I alone may not be interchangeable with the corresponding region of another wild-type superantigen although when interchanged together with other regions determining TCR binding and T cell activation, 30 the result becomes a functional active superantigen. The regions concerned normally comprise less than 20 residues, in particular for superantigens analogous to SEA. The replacing amino acid residue thus is different from the replaced residue, and conceivably includes also conserved substitutions and other 35 amino acid substitutions leading to functionally active modified superantigens allowing binding to TCRV β and activation of a

subset of T cells. This means that the inventively modified superantigens in its broadest sense encompass any modified superantigen in which one or more amino acids in the aforementioned regions have been functionally replaced.

5 The term "conserved substitution" refers to replacement of an amino acid residue by a chemically similar residue, e.g. a hydrophobic residue for a different hydrophobic residue, a charged residue for a different but similarly charged residue etc.

10 As superantigens I, II etc, the staphylococcal enterotoxins, in particular those that coordinate zinc, were at the priority date preferred, i.e. SEA, SEE, SED and possibly also SEH.

15 The regions involved may have either of the above-mentioned functions (see the heading "The Discovery that has resulted in the Invention" and the Experimental Part):

1. A great impact on the superantigen activity as such and a limited effect on the TCR specificity, in particular on V β specificity. For SEA-type superantigens this means region A (amino acid positions 20-27).
- 20 2. A profound effect on the specificity with respect to binding to polymorphic TCR chains, such as the V β chain. For SEA-type of superantigens this means regions C (amino acid positions 60-62), F (amino acid positions 110-126) and H (amino acid positions 200-207).

25 For SEA-like superantigens this means one or more of the substitutions (applied to grafting from SEA to SEE; SEE/A chimeras):

Region A: R20G, N21T, S24G, R27K

Region C: G60D, P62S

30 Region F: H111R, H114Q, G115Y, F117Y, G118N, S124V, G126D

Region H: D200G, P206S, D207N

At the priority date it was preferred to carry out all substitutions for each region. For other superantigens, analogous substitutions between corresponding positions/regions 35 could conceivably also be carried out.

Typically one could start from one first superantigen, like SEE and SED, and then replace one or more of its unique $V\beta$ binding regions with the corresponding region(s) of a second superantigen (e.g. SEA), the first and second superantigens 5 preferably being selected so that the antibody titer in normal human sera for the first superantigen is lower than for the second superantigen. For SEA and SEE chimeras, the best modes correspond to the chimeras SEE/A-A, SEE/A-AH, and SEA/E-BDEG, with absolute preference for SEE/A-A. See the experimental part 10 and the figures.

Together with the regions A, C, F and H also amino acid residues at other parts can be exchanged. One type of exchange is to reduce the class II binding ability, because this property is associated with common side effects encountered in 15 superantigen therapy (general immune activation with concomitant systemic release of tumor necrosis factor (TNF) and interferon- γ). For superantigens such as SEA, SED and SEE, positions that are important for the ability to coordinate zinc ions may preferably be changed, i.e. positions 225 and 227, for 20 instance in SEA mutation H225A and in particular D227A will have a positive impact on reducing toxic side effects (see Abrahmsén et al WO9601650 and Fraser et al 1993).

Other substitution may be performed althroughout the molecule as long as they do not destroy the superantigen function, for 25 instance conserved substitutions, in particular outside regions involved in the binding to class II and TCR. A change in the DNA sequence for altering the MHC class II binding or any other change on the DNA level may be carried out either before or after the change in regions providing for binding to 30 TCR. These other types of modifications can equally well have been introduced prior to the amino acid replacement in Region I. In the context of the present invention, the concept of using a "wild-type superantigen" at the start of the modification according to the claims thus primarily refers to 35 the wild-type amino acid sequence in region I outside of which prior modifications may have taken place.

Construction of chimeric and mutated superantigens can be carried out according to techniques well-known in the art. The switch from a region specific for one superantigen to the corresponding region in another superantigen is done on the 5 genomic level and may be accomplished by replacing a complete sequence or by point mutations of those specific bases that are required to end up in the desired amino acid sequence. See for instance the experimental part and also the prior art references cited above. The term "mutation" comprises 10 replacing, inserting or removing one or more amino acid residues by modifying the DNA sequence coding for the protein to be mutated.

The superantigen to be used in the inventive method can be a non-conjugated superantigen modified as described above, i.e. a 15 modified superantigen lacking a specifically attached target-seeking moiety but with a pronounced ability to bind to both MHC class II antigens and a subset of T cells via TCR. More preferably the modified superantigen, preferably a chimeric superantigen, is conjugated to a target-seeking moiety. In the 20 latter case the preferred variants are fusions between the target-seeking moiety and the modified superantigen. The conjugates as such are novel and are a separate aspect of the invention.

25 The structures of the inventive conjugates are analogous to earlier known antibody-superantigen conjugates (Dohlisten et al WO9201470; Abrahmsén et al WO9601650, both publications hereby being incorporated by reference), i.e. the conjugates often comply with the formula:

30

$$T-B-SA(m)$$

where T represents the target-seeking moiety, SA(m) the modified, preferably chimeric, superantigen as defined above, and B is a covalent bridge linking T and SA(m) together. T may in principle contain further superantigen moieties (SA(m)), an 35 SA(m) further target-seeking moieties, although in the

preferred conjugates there is only one target-seeking moiety and one modified superantigen moiety as defined above.

T can in principle be any structure that is able to bind to cell surface structure, preferably a disease specific structure. The structure against which T is directed is usually different from (a) the V β chain epitope to which SA(m) binds, and (b) the MHC class II epitopes to which superantigens bind. The target-seeking moiety is primarily selected among interleukins (e.g. interleukin-2), hormones, antibodies including antigen binding fragments of antibodies, growth factors etc. See for instance Woodworth, Preclinical and Clinical development of Cytokine toxins presented at the conference "Molecular approaches to cancer Immunotherapy", Ashville, North Carolina, November 7-11, 1993.

15 At the priority date, it was preferred that T was an antibody (full length antibody, Fab, F(ab)₂, Fv, single chain antibody and any other antigen binding antibody fragment), with particular emphasis for antibody active fragments (such as Fab), directed towards the so called C242 epitope (Lindholm et al WO9301303) or more preferably towards the binding epitope for the lung cancer specific 5T4 antibody (Stern et al WO8907947). This, however, does not exclude that other cancer specific antibodies may function equally well or even better. The term "antibody" comprises monoclonal as well as polyclonal 25 variants, with preference for monoclonal preparations.

T may also be directed towards unique structures on more or less healthy cells that regulate or control the development of a disease.

30 The bridge B may be selected as previously described (Döhlsten et al WO9201470; and Abrahmsén et al WO9601650), i.e. B shall preferably be hydrophilic and exhibit one or more structure(s) selected among amide, thioether, disulphide etc. The most prominent bridges are those obtained by recombinant 35 techniques, i.e. the conjugation takes place at the genomic level. In such cases oligopeptide bridges containing

hydrophilic amino acid residues, such as Gln, Ser, Gly, Glu, Pro, His and Arg are preferred. Particularly preferred Bs are peptide bridges consisting of 1-10 amino acid residues, with absolute preferences for 3-7 amino acid residues. A typical 5 bridge is the tripeptide GlyGlyPro, SEQ ID NO 1.

The manufacture of the novel inventive conjugates may be carried out in principle according to two main routes: 1. Recombinant techniques and 2. Chemical linking of a target-10 seeking moiety T to a modified, preferably chimeric, superantigen (SA(m)) as defined above. These methods are well recognized for the ordinary skilled worker and comprise a large number of variants.

Chemical linking of a modified non-conjugated superantigen 15 to a target-seeking moiety T often utilizes functional groups (e.g. primary amino groups or carboxy groups) that are present in many positions in the compounds. It follows that the final product will contain a mixture of conjugate molecules differing in linking positions, as well as hetero- and homo-conjugates.

20 For recombinant conjugates (fusion proteins) the obtained conjugate substance will be uniform with respect to the linking position. Either the amino terminal of the chimeric superantigen is linked to the carboxy terminal of the target-seeking moiety or vice versa. For antibodies, such as intact 25 antibodies and antigen-binding fragments (Fab, Fv, single chain antibodies etc), either the light or the heavy chain may be utilized for fusion. At present time recombinant conjugates are preferred, with utmost preference for Fab fragments and linking of the amino terminal of the chimeric superantigen to the first 30 constant domain of the heavy antibody chain (CH1), without exclusion of the analogous linking to the light chain or to the VH and VL domain that also may give quite good results.

The main host cell for large scale recombinant production of the inventive modified superantigens (fused forms as well as 35 non-conjugated forms) is E. coli. This host provides for in principle two routes: intracellular production and secretion.

The latter variant is preferred because it offers purification of correctly folded proteins from the periplasma and from the culture medium. The above does not exclude that it is possible to produce active conjugates also in other host cells, e.g. 5 eukaryotic cells, such as yeast or mammalian cells.

PHARMACEUTICAL COMPOSITIONS, DOSAGE AND ROUTES OF ADMINISTRATION.

A third aspect of the instant invention is pharmaceutical compositions containing the inventive modified, preferably 10 chimeric, superantigens as defined above (both conjugated and non-conjugated forms). The compositions contemplated are known in the field, except that now they contain the instant inventive superantigen. Thus, the compositions may be in the form of a lyophilized particulate material, a sterile or 15 aseptically produced solution, a tablet, an ampoule etc. Vehicles such as water (preferably buffered to a physiologically acceptable pH value by for instance PBS) or other inert solid or liquid material may be present. In general terms the compositions are prepared by the conjugate being 20 mixed with, dissolved in, bound to, or otherwise combined with one or more water-soluble or water-insoluble aqueous or non-aqueous vehicles, if necessary together with suitable additive and adjuvants. It is imperative that the vehicles and 25 conditions must not adversely affect the activity of the modified superantigen.

Normally the inventive superantigen will be sold and administered in predispensed dosages, each one containing an effective amount of the conjugate that, based on the result not presented, is believed to be within the range of 10ng - 50 mg, 30 such as within 10 ng - 1 mg or within 10 µg - 50 mg. The exact dosage will vary from case to case depending on the patient's weight and age, route of administration, type of disease, target-seeking moiety, superantigen, linkage (-B-) etc.

The administration routes will be those commonly contemplated 35 within the field, i.e. a target cell killing effective amount or therapeutically active amount of a superantigen modified

according to the invention is brought into contact with the target cells. For the indications specified above this mostly means parenteral administration, such as injection or infusion (subcutaneously, intravenously, intraarterial, intramuscularly, 5 intraperitoneal) to a mammal, such as a human being. The modified, preferably chimeric, superantigens contemplated may be administered locally or systemically.

By the term "target killing effective amount" is contemplated that the amount is effective in activating and directing T 10 cells to destroy target cells.

The preferred administration route at the priority date is the same as contemplated for the superantigen conjugates according to Dohlsten et al WO9201470 and Abrahmsén et al WO9601650. This means 1-5 hours' intravenous infusion 15 (preferably 4 hours) per day combined with a fever-reducing agent (paracetamol). The administration is to be repeated during some days, for instance 4 days, with care consideration taken for the risk of boosting antibodies directed towards the conjugate.

20 The inventive superantigens may be administered either as the main therapy or in preferred modes as adjuvant therapy in connection with surgery or other drugs.

In the context of therapy we have found that antibody 25 preparations that are pure with respect to non-covalently associated heavy and light antibody chains provide advantages over preparations that contains antibodies in which the chains are linked together via cystine linkages. Accordingly a fourth aspect of the invention is the therapeutic use of an antibody 30 preparation, in particular an Fab preparation, in which the cysteine residues linking the chains together have been replaced by an amino acid not permitting disulfide formation, for instance serine. The most preferred antibody specificities for this aspect of the invention were at the priority date the 35 C242 mab (Lindholm et al., WO9301302) and the 5T4 mab as defined in the references cited above. In the preferred

variants one of the antibody chains is fused to a superantigen that is capable of activating a subset of T cells in a V β specific manner as described above. The superantigen may be a wild-type, a chimera, or a point-mutated version (and 5 combination thereof) as described above or by Dohlsten et al WO9201470 or by Abrahmsén et al WO9601650. This aspect of the invention also comprises pharmaceutical compositions as described above, but containing an antibody preparation as defined for this aspect of the invention instead of a chimeric 10 superantigen.

At the priority date it was preferred to use the Fab fragment 5T4 antibody (Stern et al, WO8907947) in combination with the SEE/A-A chimera with the mutation D227A. The preferred Fab 15 fragment was mutated in both chains in the position providing interchain disulfide linkage (cys to ser). In order to increase the yield of the antibody/fusion protein when produced in E coli, mutations were also carried out in the V κ chain at certain positions. See the experimental part.

20

MATERIALS AND METHODS

Construction of SEA/SEE chimeric genes

Construction of SEA/SEE chimeras were made using the polymerase chain reaction (PCR) based method, sequence overlap 25 extension (Horton et al). PCR reactions were performed with Ultima (Perkin-Elmer) according to manufacturer's recommendations. PCR produced fragments were cloned in PCR-script (Stratagene, USA) and sequenced to verify the correct sequence. The chimeric superantigen genes were then subcloned in the expression vector 30 pKP889 (Abrahmsén et al 1995), fusing the SE constructs to the heavy chain portion to the Fab fragment of the murine monoclonal antibody C215. The SEA and SEE recombinant fusion proteins were produced as full length polypeptides in accordance with the consensus sequence for signal peptide 35 cleavage (von Heijne 1986)

Protein expression and purification

The *Escherichia coli* K12 strain UL635 was used for expression of the Fab-SE fusion proteins and the SEA mutants as described earlier (Abrahmsén et al 1995). Fab-SE fusion proteins were harvested by centrifugation at 5000 g and the supernatant fractions were subjected to purification on protein G Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) as earlier described (Abrahmsén et al 1995). The purity of the affinity purified Fab-SE variants were >90% pure when analyzed by SDS-PAGE.

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Cells

The human B-cell lymphoma cell line Raji and human colon carcinoma Colo 205 were cultured in complete R-medium (RPMI-1640 supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, Ltd. Paisley Scotland) 1 mM glutamine; HyClone Europe, Ltd. Cramlington, 5×10^{-5} M β -mercaptoethanol; ICN Biomedicals INC. Costa Mesa CA, 0.1 M NaHC0₃; Seromed Biochrome, 1×10^{-2} M Hepes buffer; HyClone Europe, Ltd. Cramlington., 0.1 mg/ml gentamycine; Biological Industries Kibbutz Beit Haemek Israel, 1×10^{-3} M sodium pyruvate; HyClone Europe, Ltd. Cramlington). CHO cells transfected with human C215 and CD80 molecules were cultivated in complete R-medium supplemented with 0.5 mg/ml Geniticide (G418) Gibco BRL, Life Technologies, Ltd. Paisly Scotland). Peripheral blood mononuclear cells (PBM) were prepared from heparinized blood from normal donors. The cells were isolated by density centrifugation over Ficoll-Paque as previously described (Dohlsten et al 1991). Human T lymphocytes were purified to homogeneity by positive selection using MiniMACS columns in conjunction with magnetic beads coated with monoclonal antibodies specific for human CD4 and CD8 (Miltenyi Biotec GmbH, Germany) according to the manufacturers specifications. Human SEA and SEE reactive cell lines were generated as previously described (Dohlsten et al 1994). Human TCR V β 22 expressing cell line was generated from a primary stimulated SEA reactive cell line using positive selection with magnetic

Dynabeads (Dynal A.S., Norway) coated with TCR V β 22 specific monoclonal antibody (Immunotech, France). Enriched cells contained > 95 % TCR V β 22 $^+$ T cells as determined by flow cytometry (data not shown). Murine T-cell hybridomas (I1B3, 2B 5 and 11.40) were generated as described (Fleury et al 1991).

Cytotoxicity assay

Cytotoxicity was measured in a standard ^{51}Cr release assay after 4 or 6 hours as previously described (Dohlsten et al 10 1991). Human Colo205 or Raji cells were used as target cells. The effector cells, either SEA or SEE reactive human T cell lines or TCR V β 22 cell lines, were added at an effector to target ratio of 30:1. ^{51}Cr -labeled target cells were used in the cytotoxicity assays at 2500 cells/200 ml complete medium in 15 V-bottomed microtiter wells. C215Fab-SEA/E hybrids were added at various concentrations as indicated and ^{51}Cr release was measured in a g-counter. The percentage specific cytotoxicity was calculated as $100 \times [(c.p.m. \text{ experimental release} - c.p.m. \text{ background release}) / (c.p.m. \text{ total release} - c.p.m. \text{ background release})]$.

Lymphocyte proliferation assays

To measure proliferation 10 5 human T cell responders were incubated at 37°C with 10 4 irradiated (20.000 Rad) stimulator 25 cells in 200 ml complete medium in U-shaped 96-well microtitre plates with varying amounts of C215Fab-SEA/E hybrids for 72 hours. Proliferation was estimated by incorporation of [^3H]-thymidine as described (Dohlsten et al 1988).

30 Analysis of Fab-SAg induced IL-2 production.

Murine T-T hybridoma cells (10 5) were incubated in 200 m complete R-medium with C215Fab-SEA/E chimeric proteins in the presence of 2x10 4 Raji stimulator cells. After 48 hours supernatants were harvested and analyzed for presence of murine 35 IL-2. Briefly, cytokine content was analyzed using rat anti mouse cytokine mAb as catcher antibodies. Purified rat anti

mouse IL-2, biotin-labeled rat anti-mouse IL-2, rIL-2 was purchased from PharMingen (San Diego, CA). Biotin-labeled anti cytokine mAb, Vectastain ABC kit (Vector Laboratories, CA) and peroxidase substrate kit (Bio-Rad Laboratories, CA) were used for detection of cytokines. The absorbance was determined in ImmunoReader NJ2000 (InterMed Roskilde, Denmark) at 405 or 450 nm.

Mutation of 5T4 Fab

10 Construction of a vector for expression of 5T4Fab-SEA in *E. coli*.

The Fv-encoding portions of 5T4 were cloned from the 5T4 hybridoma, obtained from Dr Peter Stern (Stern et al., WO8907947). In more detail: cDNA was made from the mRNA, 15 regions of the entire variable domains and parts of the signal sequences as well as the first constant domain of the heavy chain and the constant domain of the light chain were amplified by PCR. The oligonucleotides

5'-CAATTTCTTGTCCACCTGGTGC-3' (SEQ ID NO: 2) and

20 5'-ACTAGTCGACATGGATGGAGCTTATCATIyTCTT-3' (SEQ ID NO 3)

were used for the heavy chain, resulting in a 553 bp fragment, while the oligonucleotides

5'-ACTAGTCGACATGGGCITCAAGATGGAGTCACAkwywCwGG-3' (SEQ ID NO:

4) and

25 5'-GCGCCGTCTAGAATTAAACACTCATTCCCTGTTGAA-3' (SEQ ID NO: 5)

were used for the light chain, yielding a 724 bp fragment. For each chain three separate clones were sequenced and found to be identical. DNA fragments suitable for insertion into the expression vector (ref) were obtained in a second PCR step. In

30 order to assemble a Fab-expression plasmid, the variable regions of 5T4 were fused to sequences coding for constant regions from the murine IgG1/k antibody C242 mab (Lindholm et al, WO9301302). A region coding for a superantigen derived from

35 staphylococcal enterotoxin A (SEA) was fused after the heavy chain. The verified sequence for the V κ chain antibody framework for the 5T4 antibody is given in the results.

Mutagenesis of 5T4

Seven amino acid replacements were introduced in the region coding for the antibody framework. These were Phe10Ser, 5 Thr45Lys, Ile63Ser, Tyr67Ser, Phe73Leu, Thr77Ser and Leu78Val. Similarly, the Cys residues in either chain involved in the interdomain disulfide bond were replaced by serine residues resulting in the mutations Cys458Ser in the heavy chain and Cys214Ser in the light chain. The mutations were introduced 10 using PCR-based mutagenesis and the DNA sequence obtained was confirmed using sequencing.

Fermentor expression and purification of 5T4Fab-SEA.

The expression plasmid contains the kanamycin resistance gene 15 and a lacUV5-promoter that may be induced with IPTG. The fusion proteins were purified from the clarified culture medium using protein G Sepharose and SP-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and formulated in citrate buffer using Sephadex G-25, essentially as described. Characterization using 20 SDS-PAGE, reverse phase HPLC and mass spectrometry showed that the purified fusion protein was more than 95 % pure and had the correct molecular mass.

RESULTS: SUPERANTIGEN MODIFICATIONS.

25 The superantigen dependent cellular cytotoxicity (SDCC) of C215Fab-SEA and of C215Fab-SEE against MHC class II⁺ Raji cells, was analyzed using SEA- and SEE-reactive human T cells as effector cell lines. Despite the difference in V β specificity between SEA and SEE both superantigens exhibited 30 induction of comparable degree of cytotoxicity with both effector cell lines (Fig. 1). To discriminate between effects of MHC class II presentation and direct effects of SEA and SEE in TCR recognition, they were examined in superantigen-antibody dependent cellular cytotoxicity (SADCC) against C215 expressing Colo205 cells. In this assay the Fab moiety directs the fusion 35 protein to C215-expressing target cells and results in the

presentation of fused SE molecules to cytotoxic T-cells (CTL) independent of MHC class II molecules (Dohlsten et al 1994). Despite >80% amino acid sequence identity between SEA and SEE the TCR interaction of SEA and SEE displays qualitative differences in this type of assay. The C215Fab-SEA fusion protein retains its ability to direct SEA and SEE reactive CTL against the MHC class II⁻ target cells (FIG 1) while C215Fab-SEE fails to induce cytotoxicity of the, MHC class II⁻ target cells, neither with SEA nor with SEE reactive CTL (FIG 1).

10 It has previously been reported by other investigators that the differences in V β specificity between SEA and SEE primarily relates to a three amino acid difference in the loop preceding and in the irregular a5 helix (Irwin et al 1992, Hudson et al 1993, Fraser et al 1993, and Mollick et al 1993). The 15 difference in respect to TCR interaction reported in this investigation is not related to altered TCR V β specificity since the ability of C215Fab-SEA to induce MHC class II independent cytotoxicity is not restricted to SEA reactive CTL but is also seen with SEE reactive CTL.

20 Sequence homology analysis of SEA and SEE (Fig. 2) reveals that the non-identical amino acid residues are concentrated to eight distinct regions. Outside these eight regions, making up to 34% of the sequence, the identity of the two SE's is 97%, with conserved amino acid substitutions accounting for the 25 remaining differences. Four of the non-homologous regions are structurally close to the two MHC class II binding sites (B, I E and G), and are not likely to interact with the TCR (Fig. 3) The additional four regions (A: AA 20-27, C: 60-62, F: 161-176 and H: 200-207) are located on the edge of the molecule (Fig. 30 3), in the vicinity of the TCR binding site, located in the groove between the two subdomains (Kappler et al 1992). To investigate the qualitative difference in TCR recognition between SEA and SEE we made hybrid proteins by grafting the regions from SEA to SEE as single region chimeras (SEE/A-A, -(35 -F, H) as double region hybrids (SEE/A-AH) and by grafting the regions located in the vicinity of the MHC class II binding

sites on SEE to SEA (SEA/E-BDEG) (Fig 4). All of the chimeric SEs were expressed as C215Fab fusion proteins to be able to detect differences with respect to their activity in the absence of MHC class II.

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The SEA/E hybrid proteins in fusion with the C215Fab moiety displays difference in Fab targeted cytotoxic assays.

The SDCC activity of C215Fab-SEE/A hybrid proteins against MHC class II⁺ Raji cells were analyzed using SEA-reactive human 10 T cells as effectors. The EC₅₀ values of all C215Fab-SE hybrids as well as the C215Fab-SEAw and -SEEwt falls in the margin of errors (e.g. 10⁻¹²-10⁻¹¹ M, Fig 5). The only detectable 15 difference is a slightly reduced plateau for the C215Fab-SEE/A-AH hybrid, indicating a loss of responding T cells. On the other hand in SADCC experiments where the cytotoxicity is 20 directed towards MHC class II⁻/C215⁺ Colo 205 cell line, only C215Fab-SEE/A-A, C215Fab-SEE/A-AH and C215Fab-SEA/E-BDEG induced comparable cytotoxicity as the C215Fab-SEAw (Fig 5). The C215Fab-SEE/A-F hybrid is able to induce C215 targeted 25 cytotoxicity at higher concentrations (EC₅₀ >10⁻¹⁰ M). Although the C215Fab-SEE/A-H hybrid is able to induce C215 targeted cytotoxicity with similar half maximal concentration as C215Fab-SEAw (e.g. EC₅₀ 10⁻¹³ M), the absolute level of cytotoxicity is strongly reduced (Fig 5). This difference could 30 be a consequence of a restricted V β specificity of the C215Fab-SEE/A-H while the ability of inducing C215 targeted cytotoxicity prevails in the responding T cell sub-population. To further investigate this notion we prepared human V β 22 oligoclonal CTL line. Human V β 22 are analogous to murine V β 3 in the respect that it is a SEA non SEE specific V β family. It has previously been shown (Mollick et al 1993) that the major 35 contribution of SEA and SEE V β is primarily residing in the three amino acid difference between SEA and SEE in region H (A 200-207). In SDCC assays against MHC class II⁺ Raji targets, using the V β 22 oligoclonal CTL line as effectors, only hybrids containing the SEA-H region are able to give C215Fab-SEAw-like

response (e.g. C215Fab-SEE/A-H, C215Fab-SEE/-AH and C215Fab-SEA/E-BDEG, Fig. 6). The C215Fab-SEE/A-A hybrid, that was able to induce a full SDCC response with whole CTL populations as effectors is in this assay strongly reduced both in half maximal concentration and in the plateau (Fig. 6). When the cytotoxicity of the V β 22 CTL is directed towards the MHC class II-/C215 $^{+}$ Colo 205 cell line only hybrids containing both SEA-F and SEA-H (e.g. C215Fab-SEE/A-AH and C215Fab-SEA/E-BDEG) regions are able to induce a cytotoxic response, comparable to a C215Fab-SEAwt (Fig. 6). The hybrid containing only the SEA region A (C215Fab-SEE/A-A) induces a lower level of cytotoxicity with a comparable EC50 value. This indicates that the remaining activity seen with the C215Fab-SEE/A-H hybrid in SADCC with the whole T cell population as effectors is not a consequence of the hybrid induced response in restricted population of T cells. A more likely explanation for the observation is that the ability to induce a SADCC response of the C215Fab SE hybrid proteins is primarily residing in the SEA-A region with a minor contribution from the SEA-H and -F regions. There is no evidence that this quality is restricted to any subset of T cells in the combined SEA-SEE responding T cell population, since C215Fab SEA is able to induce the same response with as well with SEE reactive CTLs and C215Fab-SEE/A-A is able to fully to reconstitute the response seen with C215Fab-SEA.

The SEA/E hybrid proteins in fusion with the C215Fab moiety displays difference in Fab targeted proliferation assays.

It has been previously shown that purified resting human T cells are induced to proliferate by presentation of C215Fab-SE on a MHC class II-/C215 $^{+}$ /CD80 $^{+}$ cell line (Lando et al 1993). The ability of C215Fab-SEA to induce MHC II independent proliferation is however markedly reduced with C215Fab-SEE (Tab. 1). To investigate if this difference in quality shows the same confinement to SEA region A, as was seen with SADCC, we investigated the proliferative capacity of C215Fab-SE

hybrids, presented by either CHO-DR1⁺/CD80⁺ or CHO-C215⁺/CD80⁺ transfected cell lines, on purified resting human T cells. When presenting the Fab-SE conjugates on CHO-DR1⁺/CD80⁺ no differences between the different SE proteins were noted (data not shown). However grafts of SEA region A, C and H in SEE potentiate the proliferative activity compared to C215Fab-SEE. The best results were obtained by grafting SEA regions A and C indicating an important role for region A as was seen for the MHC class II independent cytotoxicity. By using a negative selection it is possible that the differences between Fab-SEA and -SEE would be more prominent.

v β specificity of SE-hybrids

To further investigate if the C215Fab-SEA/SEE hybrid-fusion proteins were associated with a certain v β specificity we used SEA reactive murine T cell hybridomas expressing v β 1, v β 3 and v β 11. It is obvious from the data obtained that all of the regions investigated, directly or indirectly, affects the interaction with the TCR. By grafting SEA regions C and F in C215Fab-SEE the activity towards the SEA and SEE cross reactive v β 1 hybridoma I1B3 is destroyed. The same chimeras seems to have no or minor effects on the activity of v β 3 and v β 11 hybridomas (2.B4 and 11.40) in comparison with C215Fab-SEE. By grafting SEA region A in C215Fab-SEE the activity towards v β 3 (2.B4) is enhanced by at least a factor 100, in comparison to C215Fab-SEE. More pronounced effects are seen with the same cell line by grafting SEA region H in C215Fab-SEE. This pronounced effect on the influence of v β 3 specificity by SEA region H has also been noted by earlier investigations (Mollie et al 1993). The same chimera however (C215Fab-SEE/A-H), seems to reduce the activity towards the SEA/SEE cross reactive v β 1 and v β 11 hybridomas (I1B3 and 11.40) by a factor 10. In conclusion, the TCR interaction of SEA seems to involve all of the SEA-SEE, variable regions A, C, F and H.

Seroreactivity

The seroreactivity in human serum samples towards the chimeric SEs was investigated both in pooled samples from different parts of the world as well as in individual serum samples. By grafting both SEA regions A and H in SEE we obtained an intermediate seroreactivity (Fig 8). A similar seroreactivity was also seen against the chimera C215Fab-SEE/A. However, single grafts of SEA region A in SEE (C215Fab-SEE/A-A) gave a C215Fab-SEE like seroreactivity, indicating that SEA region H is responsible for the remaining seroreactivity against C215Fab-SEE/A-AH. This indicates that the SEA region is part of dominating antigenic epitope in SEA. The seroreactivity from pooled serum samples from other parts of the world (Japan and USA) as well as 14 individual samples from Sweden all confirms the same general pattern (data not shown).

Results: Mutations of the Fab part of the fusion proteins.**Expression of 5T4FabSEA-constructs**

The production level in *E.coli* of 5T4Fab-SEA in the fermenter was found to be significantly lower than other Fab-superantigens previously studied in our lab. Two types of modifications were therefore introduced to increase the production level. Firstly, seven different point mutations in the framework region of the light chain were introduced. These were Phe10Ser, Thr45Lys, Ile63Ser, Tyr67Ser, Phe73Leu, Thr77Ser and Leu78Val. Secondly, the cysteine residues making the disulfide bond connecting the heavy and the light chains were replaced by serine residues. The latter modification resulted in a three-fold increase and the 7 point mutations in an additional 12-fold increase in the production level. In addition to the significantly increased production level, removing the disulfide bond also gives a more homogenous product since the possibility of the these reactive thiol groups to react with other thiol containing agents is excluded. The modified 5T4 molecule was checked for affinity to its antigen as well as for biological activity in SADCC. No

differences between the mutant form and the wildtype form could be detected in these assays.

The Cys/Ser mutation was also performed in the heavy and light chains of the Fab fragments of several other monoclonal antibodies. The products became homogenous and fully retained the antigen binding capability.

Sequence of region of the antibody frame work for the 5T4 V κ chain:

10 DAVMTQTPTF LLVSAGDRV~~T~~ **ITCKASQSVS** NDVAWYQQKP GQSPTLLISY 50
TSSRYAGVPD RFIGSGYGTD FTFTISTLQA EDLAVYFCQO DYNSPTFGG 100
 GTKLEIK (SEQ ID NO 6)

Underlined sequences are CDRs. Bold-typed positions were mutated: Phe10Ser, Thr45Lys, Ile63Ser, Ile63Thr, Tyr67Ser, 15 Phe73Leu, Thr77Ser, Leu78Val.

	Proliferation EC ₅₀ (pM)
C215Fab-SEAwt	2.2
C215Fab-SEEwt	6.9
C215Fab-SEE/A-A	0.9
C215Fab-SEE/A-C	2.8
C215Fab-SEE/A-F	5.7
C215Fab-SEE/A-H	1.0
C215Fab-SEE/A-AH	0.3
C215Fab-SEA/E-BDEG	1.6

Table 1.

20

	I1B3 (MuV β 1) EC ₅₀ (nM)	2.B4 (MuV β 3) EC ₅₀ (nM)	11.40 (MuV β 11) EC ₅₀ (nM)
C215Fab-SEA	10	3	0.05
C215Fab-SEE	10	>1000	0.05
C215Fab-SEE/A-A	10	10	0.05
C215Fab-SEE/A-C	>1000	>1000	0.05
C215Fab-SEE/A-F	>300	>300	0.05
C215Fab-SEE/A-H	100	3	0.3
C215Fab-SEE/A-AH	10	3	0.3

Table 2.

Figure legends**Figure 1. MHC class II dependent and independent cytotoxicity with human SEE and SEA CTL.**

MHC class II dependent cellular cytotoxicity (A and B) and C215 dependent cellular cytotoxicity (C and D) with C215Fab-SEA (■) and C215Fab-SEE (◆) as effector molecules. Cytotoxicity was analyzed in a standard 4-h ^{51}Cr release assay using a SEE-reactive human T-cell line (A and C) a SEA-reactive human T-cell line (B and D). Target cell lines were MHC class II/ C215 Raji (A and B) and MHC class II/ C215 Colo 205 (C and D). Data are from single assays that is representative of two (A and C) to five (B and D) independent experiments.

Figure 2. Homology alignment of SEA and SEE.

SEA/SEE variable regions close to the TCR binding site (A, C, F and H) and variable regions close to the two MHC class II binding sites.

Figure 3. Cartoon model of SEA.

Molscript model (Kraulis, 1991) of the SEA crystal (Schad et al. 1995). SEA/SEE variable regions close to the TCR binding site (A, C, F and H) and variable regions close to the two MHC class II binding sites. The zinc ion is a round ball.

Figure 4. Schematic representation of chimeric SE molecules.

Stretches of SEA sequence are depressed. SEA/SEE variable regions are represented by A, B, C, D, E, F, G and H.

Figure 5. MHC class II dependent and independent cytotoxicity with human SEA reactive CTL.

(A) MHC class II dependent cellular cytotoxicity and (B) C215 dependent cellular cytotoxicity of C215Fab-SEE/A-A (♦), C215Fab SEE/A-C (□), C215Fab-SEE/A-F (◊), C215Fab-SEE/A-H (●), C215Fab SEE/A-AH (Δ) and C215Fab-SEA/E-BDEG (○). Cytotoxicity was analyzed in standard 4-h ⁵¹Cr release assay using a SEA-reactive human T-cell line. Target cell lines were MHC class II⁺ C215⁺ Raji (A) and MHC class II⁺ C215⁺ Colo 205 (B). Data are from 10 single assays that is representative of five independent experiments.

Figure 6. MHC class II dependent and independent cytotoxicity with human Vb22⁺ CTL.

15 (A) MHC class II dependent cellular cytotoxicity and (B) C215 dependent cellular cytotoxicity C215Fab-SEA (■), C215Fab-SEE (◆), C215Fab-SEE/A-A (♦), C215Fab-SEE/A-C (□), C215Fab-SEE/A-F (◊), C215Fab-SEE/A-H (●), C215Fab-SEE/A-AH (Δ) and C215Fab-SEA/E-BDEG (○) as effector molecules. Cytotoxicity was 20 analyzed in a standard 4h ⁵¹Cr release assay using a Vb22 selected SEA-reactive human T-cell line. Target cell lines were MHC class II⁺ C215⁺ Raji ((A) and MHC class II⁺ C215⁺ Colo 205 (B). Data are from single assays that is representative of two independent experiments.

25

Figure 7. MHC class II dependent and independent proliferation (not in the priority applications).

Effects of Fab-SE hybrids on MHC class II dependent (A) and independent (B) T cell proliferation. Purified human T-cells 30 were stimulated for 96h with C215Fab-SEA (■), C215Fab-SEE (◆),

C215Fab-SEE/A-A (•), C215Fab-SEE/A-C (□), C215Fab-SEE/A-F (◊), C215Fab-SEE/A-H (●), C215Fab-SEE/A-AH (Δ) and C215Fab-SEA/E-BDEC (○) presented on MHC class II⁺/C215⁺ CHO-DR4/C215 transfectants (A) and on MHC class II⁺/C215⁺ CHO-CD80/C215 transfectants (B). After 72h the cells were pulsed with [³H]-thymidine for 24h and incorporated label were measured and represented as half maximal concentration (EC₅₀). Data are from a single assays that is representative of two independent experiments.

10

Figure 8. Seroreactivity in a human Ig pool.

Pool of >5000 sera from healthy donors in Southern Europe against C215Fab-SE fusion proteins. Serially diluted human Ig was allowed to interact for 1h at room temperature with 15 C215Fab-SEAw, C215Fab-SEEwt, C215Fab-SEE/A-A, C215Fab-SEE/A-H and FabSEE/A-AH. Immobilized to the micro titer plates at a concentration of 1 ng/well. Correction for C215Fab binding to serum proteins was made by subtracting the OD-value for C215Fa at each point. Each point represents the mean of duplicate 20 samples. For further details see Materials and Methods.

Table 1. Purified human T-cells were stimulated for 96h with respective C215Fab-SE presented on MHC class II negative CHO-CD80/C215 transfectants. After 72h the cells were pulsed with 25 ³H-thymidine for 24h and incorporated label was measured and represented as half maximal concentration (EC₅₀).

Table 2. Murine T cell hybridomas were stimulated for 48h with native or chimeric Fab conjugated superantigen. Activity was

measured as IL-2 production and represented as half maximal concentration (EC₅₀).

Work during the priority year.

5 In an attempt to minimize the toxicity of the superantigen chimer antibody fusion C242Fab-SEE/A-A, the chimer SEE/A-A has been mutated in Class II binding sites as described in WO9601650 and fused to C215Fab. The constructions are C215Fab-SEE/A-A-D227A, C215Fab-SEE/A-A-F47A/D227A, C215Fab-SEE/A-A-
10 H187A/D227A, C215Fab-SEE/A-A-W130A/D227A, C215Fab-SEE/A-A-D70A/D227A, C215Fab-SEE/A-A-N50S/D227A, C215Fab-SEE/A-A-N50S/D70A/D227A, C215Fab-SEE/A-A-F47Y/D227A och C215Fab-SEE/A-A-D70R/D227A. All fusions have been tested for their ability to induce proliferation of human PBMC in order to identify mutants:
15 having a lowered activity compared to C215Fab-SEE/A-A-D227A. A lowered proliferative activity has been observed for C215Fab-SEE/A-A-F47A/D227A, C215Fab-SEE/A-A-F47Y/D227A och C215Fab-SEE/A-A-D70R/D227A. Some of the chimeric fusions have also been tested for antibody titer in human normal serum (C215Fab-SEE/A-
20 A-D227/A, C215-FabSEE/A-A-D70A/D227A and C215Fab-SEE/A-A-D70R/D227A). A comparison was made with C215Fab-SEA-D227A and C215Fab-SEE-D227A. Relative C215FabSEA-D227A, the titer was much lower for each chimer tested. Relative C215Fab-SEE-D227A, the titer was slightly higher for each chimer tested.
25 This means replacements at positions corresponding to one or more, preferably two, of the positions 47, 50, 70, 130, 187, 227 as defined in sequence ID nos 7 and 8 in figure 2.

Because many varying and different embodiments may be made
30 within the scope of the inventive concept herein taught, and

because modifications may be made in the embodiments herein detailed in accordance with the descriptive requirements of the law, it is to be understood that the details herein are to be interpreted as illustrative and not in a limiting sense.

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5 Terman et al (1991) WO9110680 (patent application). Tumor killing effects of enterotoxins and related compounds.

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10 von Heijne, G (1986). A new method for predicting signal sequence cleavage sites. Nucleic Acid Res, 14, 1483-1490.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Gly Pro
1

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAATTTCTT GTCCACCTTG GTGC

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTAGTCGAC ATGGATGGAG CTITATCATI YTCTT

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTAGTCGAC ATGGGCITCA AGATGGAGTC ACAKWYYCWG G

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGCCGTCTA GAATTAACAC TCATTCCGTG TGAA

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asp	Ala	Val	Met	Thr	Gln	Thr	Pro	Thr	Phe
1				5					10
Leu	Leu	Val	Ser	Ala	Gly	Asp	Arg	Val	Thr
				15					20
Ile	Thr	Cys	Lys	Ala	Ser	Gln	Ser	Val	Ser
				25					30
Asn	Asp	Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro
				35					40
Gly	Gln	Ser	Pro	Thr	Leu	Leu	Ile	Ser	Tyr
				45					50
Thr	Ser	Ser	Arg	Tyr	Ala	Gly	Val	Pro	Asp
				55					60
Arg	Phe	Ile	Gly	Ser	Gly	Tyr	Gly	Thr	Asp
				65					70

Phe	Thr	Phe	Thr	Ile	Ser	Thr	Leu	Gln	Ala
				75					80
Glu	Asp	Leu	Ala	Val	Tyr	Phe	Cys	Gln	Gln
				85					90
Asp	Tyr	Asn	Ser	Pro	Pro	Thr	Phe	Gly	Gly
				95					100
Gly	Thr	Lys	Leu	Glu	Ile		Lys		
				105					

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser	Glu	Lys	Ser	Glu	Glu	Ile	Asn	Glu	Lys	Asp	L
				5					10		
Arg	Lys	Lys	Ser	Glu	Leu	Gln	Gly	Thr	Ala	Leu	G
		15					20				
Asn	Leu	Lys	Gln	Ile	Tyr	Tyr	Tyr	Asn	Glu	Lys	A
				30						35	
Lys	Thr	Glu	Asn	Lys	Glu	Ser	His	Asp	Gln	Phe	L
			40					45			
Gln	His	Thr	Ile	Leu	Phe	Lys	Gly	Phe	Phe	Thr	A
			50			55					6

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser	Glu	Lys	Ser	Glu	Glu	Ile	Asn	Glu	Lys	Asp	Leu
				5					10		
Arg	Lys	Lys	Ser	Glu	Leu	Gln	Arg	Asn	Ala	Leu	Ser
		15					20				
Asn	Leu	Arg	Gln	Ile	Tyr	Tyr	Tyr	Asn	Glu	Lys	Ala
					30					35	
Ile	Thr	Glu	Asn	Lys	Glu	Ser	Asp	Asp	Gln	Phe	Leu
			40					45			
Glu	Asn	Thr	Leu	Leu	Phe	Lys	Gly	Phe	Phe	Thr	Gly
	50					55					60
His	Pro	Trp	Tyr	Asn	Asp	Leu	Leu	Val	Asp	Leu	Gly
				65					70		
Ser	Lys	Asp	Ala	Thr	Asn	Lys	Tyr	Lys	Gly	Lys	Lys
		75					80				
Val	Asp	Leu	Tyr	Gly	Ala	Tyr	Tyr	Gly	Tyr	Gln	Cys
85					90					95	
Ala	Gly	Gly	Thr	Pro	Asn	Lys	Thr	Ala	Cys	Met	Tyr
			100					105			
Gly	Gly	Val	Thr	Leu	His	Asp	Asn	Asn	Arg	Leu	Thr
	110					115					120
Glu	Glu	Lys	Lys	Val	Pro	Ile	Asn	Leu	Trp	Ile	Asp
				125					130		

P A T E N T C L A I M S

1. A conjugate between
 - a) a wild-type superantigen that has been modified, and
 - b) a target seeking moiety;
- 5 wherein said modified superantigen comprises an amino acid sequence of a first wild-type superantigen, in which one or more amino acid residues in at least one region
 - i) which is present within said amino acid sequence of said first wild-type superantigen and
 - 10 ii) which functions in determining binding to TCR and activation of a subset of T cells have been replaced by a different amino acid residue,
said modified superantigen retaining an ability to activate 15 a subset of T cells.
2. The conjugate of claim 1, wherein said at least one region functions in determining binding to TCRV β .
- 20 3. The conjugate of anyone of claims 1-2, wherein said wild-type superantigen is SEA, SED, or SEE or analogous superantigens.
- 25 4. The conjugate of anyone of claims 1-3, wherein said wild-type superantigen is SEE and said at least one region is selected from the group consisting of region A, C, F and H as defined in Sequence ID no 8.
- 30 5. The conjugate of anyone of claims 1-4, wherein said wild-type superantigen is SEE in which the following amino acid residues substitutions have been made: R20G, N21T, S24G and R27K where the positions are as defined in Sequence ID no 8 in figure 2.
- 35 6. The conjugate of anyone of claims 1-5, wherein the modified superantigen is a chimera between

a) said first wild-type superantigen and
b) one or more additional wild-type superantigens,
wherein in each of said one or more amino acid residues in
said at least one region of said first wild-type
superantigen has been replaced with a corresponding amino
acid residue present in the corresponding region in said one
or more additional wild-type superantigens.

5

7. The conjugate of claim 6, wherein said first and additional
10 wild-type superantigens are selected from the group
consisting of SEA, SED, SEE and analogous superantigens.

8. The conjugate of claim 7, wherein said at least one region
15 is selected from the group consisting of regions
corresponding to A, C, F and H as defined in figure 2,
sequence ID nos 7 or 8.

9. The conjugate of claim 8, wherein at least one amino acid
20 residues at positions corresponding to 20, 21, 24 and 27 in
region A as defined in sequence ID nos 7 and 8, figure 2,
have been replaced with the corresponding amino acid
residues in region A of said one or more additional wild-
type superantigens.

25 10. The conjugate of anyone of claims 6-9, wherein said first
wild-type superantigen is SEE and said one or more
additional wild-type superantigen is SEA.

11. The conjugate of anyone of claims 7-10, wherein the
30 replacements of said one or more amino acids are at
positions corresponding to 20, 21, 24, 27 (for region A)
and/or 60, 62 (for region C) and/or 111, 114, 115, 117, 118,
124, 126 (for region F) and/or 200, 206, 207 (for region H)
as defined in figure 2, sequence ID nos 7 and 8, with
35 preference for the residue in all four positions in region A

being replaced in case said first superantigen is SEE and said one or more additional superantigens is SEA.

12. The conjugate of anyone of claims 1-11, wherein said wild-type superantigens have been selected from those requiring zinc ion for binding to MHO Class II antigens and wherein said first wild-type superantigen when present in the conjugate has a sequence in which an amino acid residue in the position involved in binding to zinc ion is replaced to the effect of a decrease in this binding ability, with emphasis for positions 225 and/or 227 as defined in figure 2, sequence ID nos 7 and 8.

13. The conjugate of anyone of claims 1-12, wherein said target-seeking moiety is an antibody, in particular an antibody active fragment.

14. The conjugate of anyone of claims 1-13, wherein said target-seeking moiety is an Fab fragment.

15. A method of treating a disease in a mammal by activating the immune system of said mammal, which method comprises administering to the mammal a therapeutically effective amount of a superantigen comprising

a first wild-type superantigen that has been modified by replacing one or more amino acid residues in at least one region which

i. is present in the amino acid sequence of said first wild-type superantigen and
ii. functions in determining binding to TCR and subsequent activation of a subset of T cells.

16. The method of claim 16, wherein said modified superantigen is a chimeric superantigen between said first wild-type superantigen and one or more additional wild-type

superantigens, in the sense that each of said one or more amino acid residues in said at least one region of said first wild-type superantigen has been replaced with a corresponding amino acid residue present in the 5 corresponding region of said one or more additional wild-type superantigens.

17. The method of anyone of claims 15-16, wherein the disease is associated with cells expressing a surface target 10 structure which bind to the superantigen at an epitope structurally different from the epitope binding to TCR and which binding to a surface binding structure allows for binding to TCR with subsequent activation of a subset of T cells.

15

18. The method of anyone of claims 15-17, wherein the disease is selected from the group consisting of cancers, viral infections, parasitic infestations and autoimmune diseases.

20 19. The method of anyone of claims 15-18, wherein said wild-type superantigens are selected from the group consisting of SEA, SED, SEE and analogous superantigens.

25 20. The method of claim 19, wherein said at least one region is selected from the group consisting of regions corresponding to regions A, C, F or H as defined in figure 2, Sequence ID Nos 7 and 8.

21. The method of anyone of claims 15-20, wherein said first 30 wild-type superantigen is SEE and said at least one region is selected from the group consisting of region A, C, F and H as defined in Sequence ID no 8, figure 2.

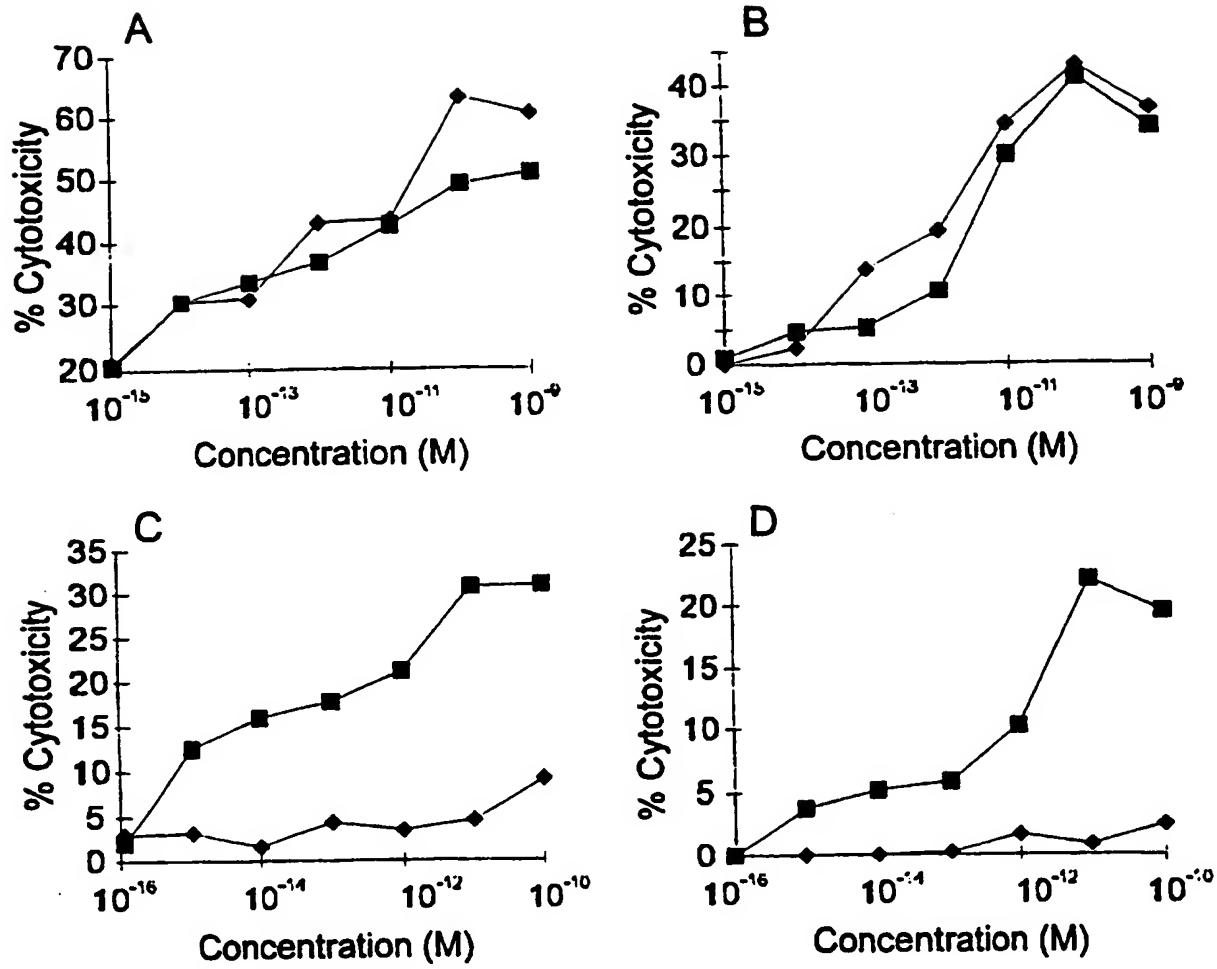
22. The method of claim 21, wherein said at least one region is 35 region A and wherein the following amino acid residues substitutions have been made: R20G, N21T, S24G and R27K

where the positions are as defined in Sequence ID no 8 in figure 2.

23. The method of anyone of claims 15-22, wherein said at least 5 one additional wild-type superantigen is SEA.
24. The method of claim 15-23, wherein said superantigen is a conjugate as defined in anyone of claims 1-14.
- 10 25. The method of claim 24, wherein the wild-type superantigen: have been selected among those that require binding to zinc ions for binding to MHC Class II and that an amino acid residue in the sequence of the first wild-type superantigen has been replaced in a position involved in binding to zinc 15 in order to lower the MHC class II binding ability.
26. The method of claim 25, wherein said position corresponds to 227 as defined in figure 2, sequence ID nos 7 and 8, with emphasis of the mutation D227A.
- 20 27. The method according to anyone of claims 24-26, wherein the target-seeking moiety is an Fab fragment in which each cysteine residue providing interchain linkage has been replaced with an amino acid residue not enabling interchain 25 linkage, with preference for serine being the replacing amino acid residue.
28. A pharmaceutical composition comprising a modified antibody in which cysteines providing for interchain cystine linkage 30 in the native antibody have been replaced to prohibit cystine formation.
29. The pharmaceutical composition of claim 28, wherein the replacing residue is a serine residue.

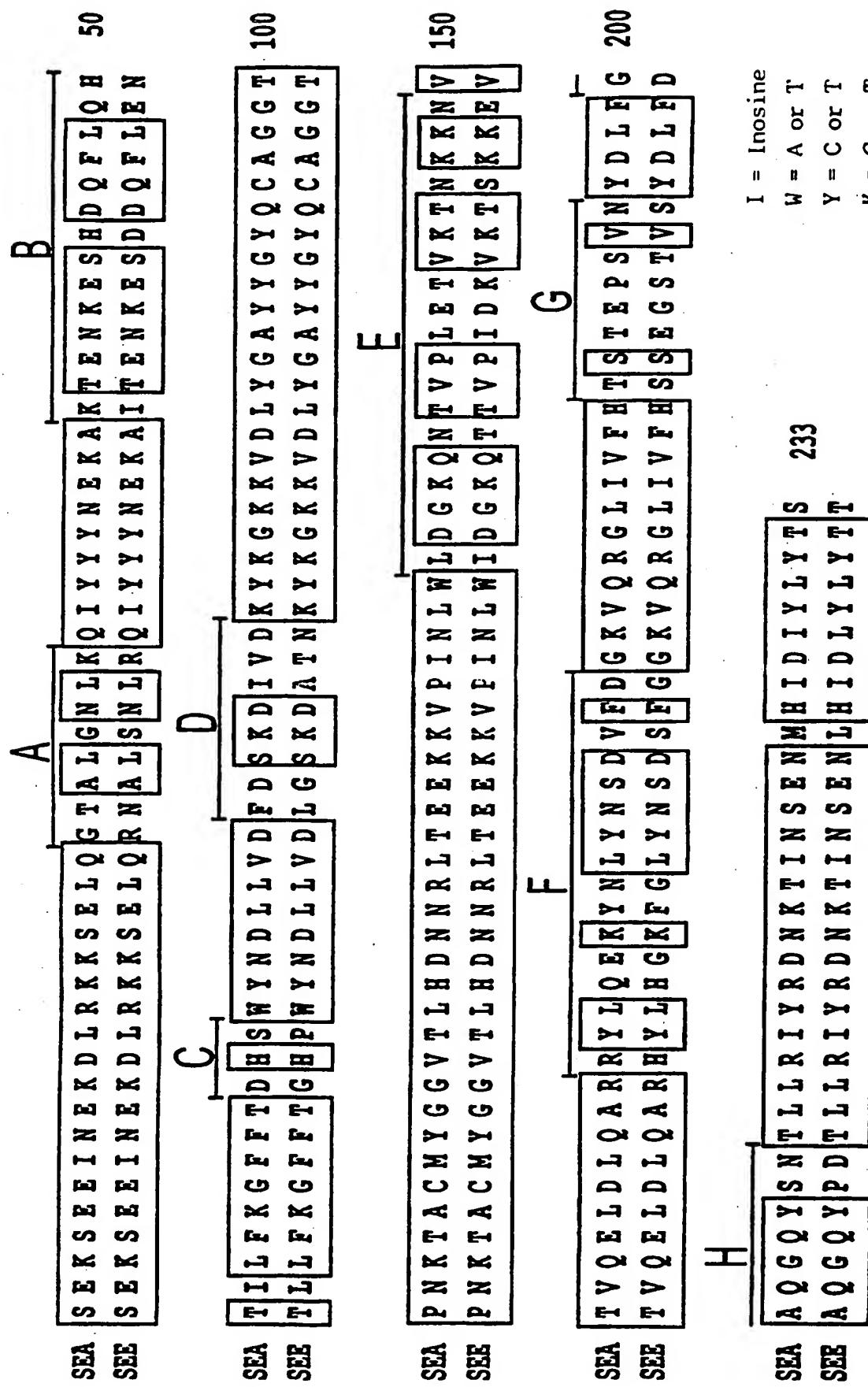
30. The pharmaceutical composition of claim 29, wherein the antibody is an Fab fragment.
31. The pharmaceutical composition of claim 30, wherein the antibody is fused to a peptide moiety providing activation of T-cells in a V β specific manner.
5

FIGURE 1



2/6

Figure 2 SEQUENCE ID NO 7: SEA SEQUENCE ID NO 8: SEE



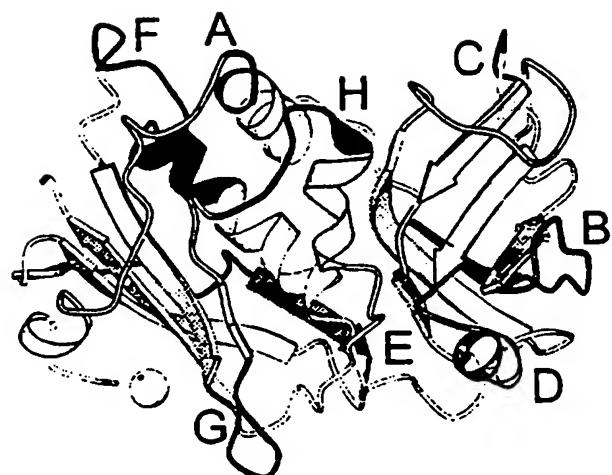


Figure 3.

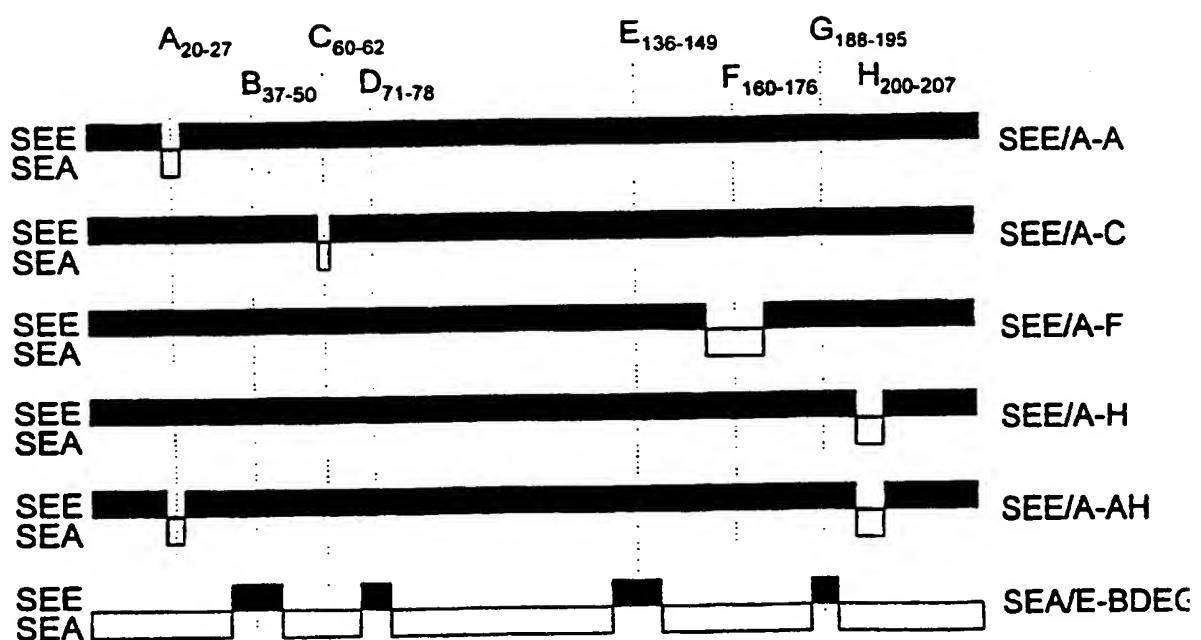


Figure 4.

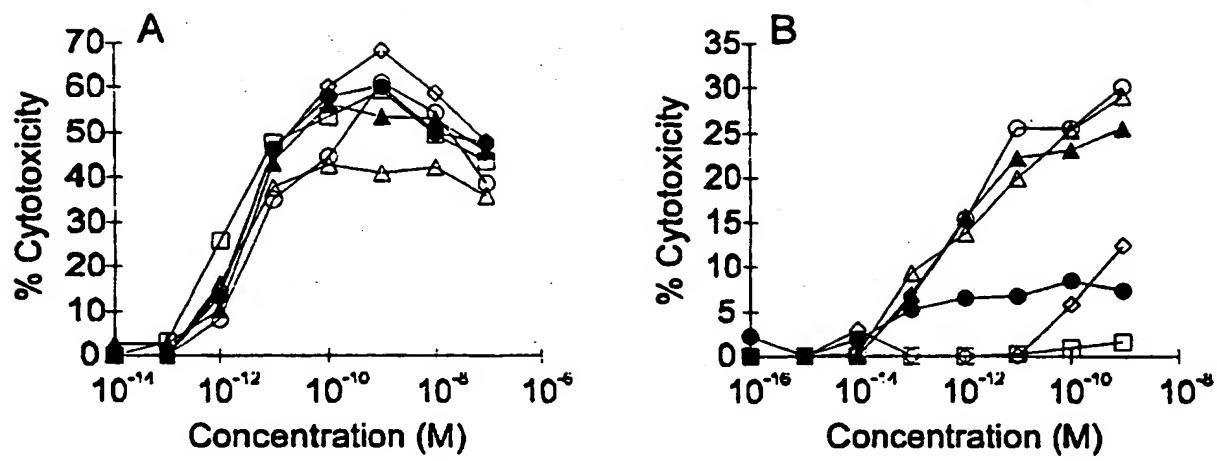


Figure 5.

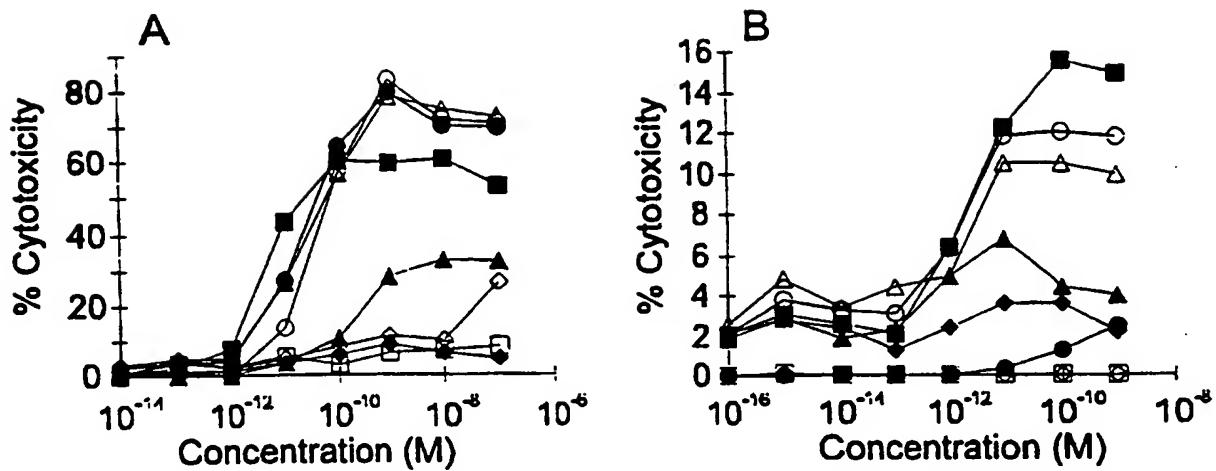
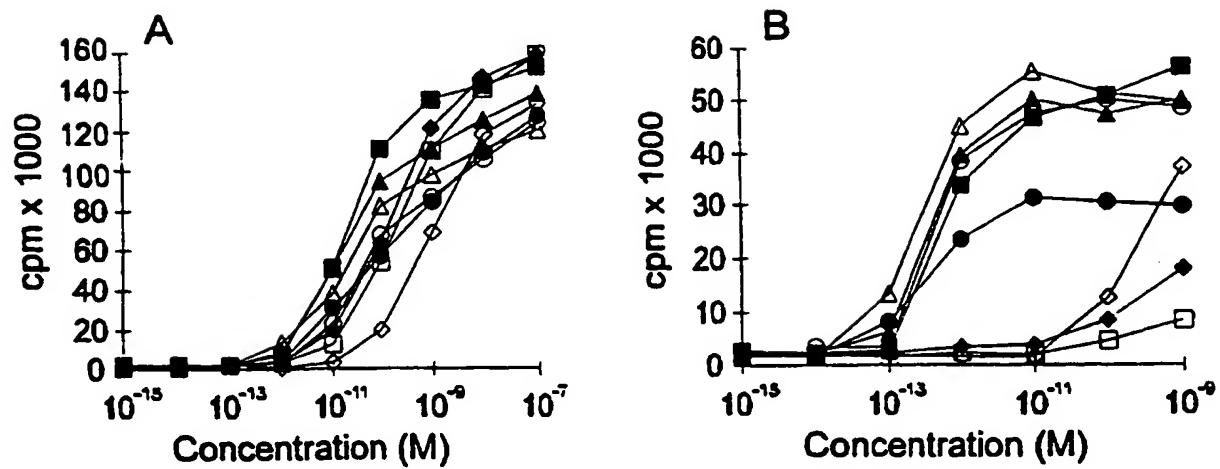
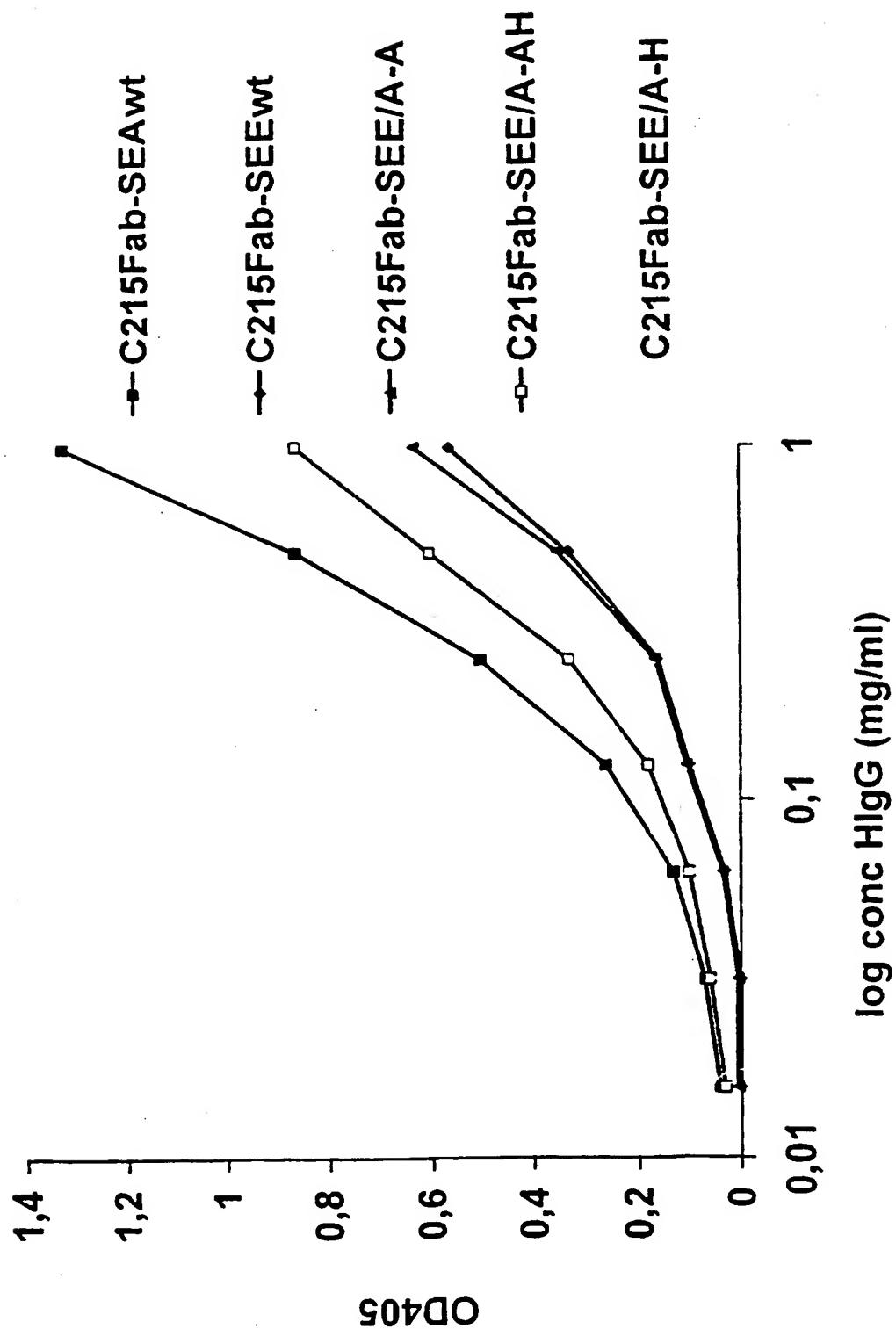
**Figure 6.****Figure 7.**

Figure 8.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00537

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 19/00, A61K 47/48, A61K 39/395, C07K 14/31
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SCISEARCH, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim
X	WO 9601650 A1 (PHARMACIA AB), 25 January 1996 (25.01.96), see tables 1-3 --	1-26
A	J. Exp. Med., Volume 175, February 1992, John W. Kappler et al, "Mutations Defining Functional Regions of the Superantigen Staphylococcal Enterotoxin B", page 387 - page 396, see figure 2 and "Discussion" --	1-11,13-2
A	J. Exp. Med., Volume 177, February 1993, Joseph A. Mollick et al, "Localization of a Site on Bacterial Superantigens That Determines T Cell Receptor beta Chain Specificity", page 283 - page 293, see table 1 --	1-11,13-2

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or date and not in conflict with the application but cited to under the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention can be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention can be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

27 August 1997

Date of mailing of the international search report

27.08.97

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM

Authorized officer

Carl-Olof Gustafsson

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00537

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim 1
A	J. Exp. Med., Volume 177, January 1993, Keith R. Hudson et al, "Two Adjacent Residues in Staphylococcal Enterotoxins A and E Determine T Cell Receptor Vbeta Specificity" page 175 - page 184 --	1-11,13-25
A	National Library of Medicine, file Medline, Medline accession no. 95136241, Ihle J: "Antibody-targeted superantigens induce lysis of major histocompatibility complex class II-negative T-cell leukemia lines", Cancer Res 1995 Feb 1;55(3):623-8 --	1-25
A	National Library of Medicine, file Medline, Medline accession no. 95385031, Holzer U et al: "Superantigen-staphylococcal- enterotoxin-A-dependent and antibody- targeted lysis of GD2-positive neuro- blastoma cells", Cancer Immunol Immunother 1995 Aug; 41(2):129-36 --	1-25
A	National Library of Medicine, file Medline, Medline accession no. 95347324, Abrahmsen L et al: "Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A", EMBO J 1995 Jul 3;14(13):2979-86 --	1-27
X	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 06620856, Medline accession no. 91355698, Gillies SD et al: "Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumor specificities"; Hum Antibodies Hybridomas 1990, 1 (1) p47-54 --	27-31
X	Proc. Natl. Acad. Sci., Immunology, Volume 90, January 1993, M. Better et al, "Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab' and F(ab')2" page 457 - page 461 --	27-31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00537

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim
X	EP 682039 A1 (BRISTOL-MYERS SQUIBB COMPANY), 15 November 1995 (15.11.95), see fig. 1 and example 2 --	27-31
A	WO 9601650 A1 (PHARMACIA AB), 25 January 1996 (25.01.96) --	31
A	Dialog Information Services, file 34, SciSearch, Dialog accession no. 13524601, Mikolajczyk SD et al: "High-field, site-specific coupling of N-terminally modified beta-lactamase to a proteolytically derived single-sulphydryl murine fab", Bioconjugate Chemistry, 1994, V5, N6, P636-646 --	27,28,30
A	US 3903262 A (ALBERT R. PAPPENHAGEN ET AL), 2 Sept 1975 (02.09.75), see column 3-4 and claims --	27
A	US 5541087 A (KIN-MING LO ET AL), 30 July 1996 (30.07.96), see column 10, lines 9-27 -----	27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00537

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reason

1. Claims Nos.: 15-27
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 15-27 relates to a method of treatment of the human or animal body by surgery or by therapy/diagnostic method practised on the human or animal body/Rule 39.1(iv). Nevertheless, a search has been executed for this claims. The search has been based on the alleged effects of the conjugates.
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00537

The present application refers to two a priori independent inventions:

1. Conjugates of a target seeking moiety and a modified wild type superantigen and a method of treating a disease with the aid of these conjugates, according to claims 1-26.
2. A pharmaceutical composition comprising an antibody lacking at least some cysteines that provide native interchain cysteine linkages, conjugates thereof and a method of treatment with such conjugates according to claims 27-31.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/SE 97/00537

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9601650 A1	25/01/96	AU 2994095 A		09/02/96
		CA 2194673 A		25/01/96
		EP 0766566 A		09/04/97
		FI 970100 A		10/01/97
		IL 114445 D		00/00/00
		NO 970108 A		20/02/97
		PL 318162 A		26/05/97
		SE 9402430 A		12/01/96
		ZA 9505746 A		20/02/96
EP 682039 A1	15/11/95	NONE		
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		CA 2194673 A		25/01/96
		EP 0766566 A		09/04/97
		FI 970100 A		10/01/97
		IL 114445 D		00/00/00
		NO 970108 A		20/02/97
		PL 318162 A		26/05/97
		SE 9402430 A		12/01/96
		ZA 9505746 A		20/02/96
US 3903262 A	02/09/75	AT 321466 B		10/04/75
		CA 990647 A		08/06/76
		DE 2311333 A,C		27/09/73
		FR 2183679 A,B		21/12/73
		JP 1045762 C		28/05/81
		JP 48103723 A		26/12/73
		JP 55038931 B		07/10/80
US 5541087 A	30/07/96	AU 3676595 A		29/03/96
		CA 2199830 A		21/03/96
		EP 0782625 A		09/07/97
		WO 9608570 A		21/03/96
		WO 9608589 A		21/03/96

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